Title

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Journal

The Journal of experimental medicine, 177(4)

ISSN

0022-1007

Authors

Rassenti, LZ
Kipps, TJ

Publication Date

1993-04-01

DOI

10.1084/jem.177.4.1039

Peer reviewed

Lack of Extensive Mutations in the V_H5 Genes Used in Common B Cell Chronic Lymphocytic Leukemia

By Laura Z. Rassenti and Thomas J. Kipps

From the Division of Hematology and Oncology, Department of Medicine, and the Sam and Rose Stein Institute for Research on Aging, University of California, San Diego, La Jolla, California 92093-0663

Summary

B cell chronic lymphocytic leukemia (CLL) is a malignancy of the $CD5^+$ B cells. Prior studies indicated that CLL B cells generally express immunoglobulin (Ig) V_H and V_L genes with little or no somatic mutations. However, a recent report indicated that V_H251, one of three V_H genes belonging to the V_H5 subgroup (e.g., V_H251, V_H32, and V_H15), not only is frequently rearranged in this disease, but also has extensive and selective mutations when expressed by CLL B cells. The extent and nature of these mutations contrasts markedly from the low level of mutations noted in V_H5 genes used by normal B cells or other Ig V genes found expressed in CLL. To determine whether this difference reflects a unique property of V_H251 or a previously unrecognized subgroup of CLL, we examined for V_H5 Ig gene rearrangements in leukemia cells from 68 patients that satisfied clinical and diagnostic criteria for $CD5^+$ B cell CLL. Southern blot hybridization studies with probes for V_H251 and the J_H locus revealed that only 7 (10%) of the 68 monoclonal CLL cell populations had undergone Ig gene rearrangement involving V_H5 genes. Two (3%) were found to have functionally rearranged V_H5 genes that shared ≥98% sequence homology with 5-2R1, a V_H251 gene isolated from a pre-B cell acute lymphocytic leukemia. The other five CLL (7%) had functionally rearranged V_H5 genes that each shared ≥99% nucleic acid sequence homology with a germline V_H32 isolated from human sperm DNA. These data indicate that V_H251 or V_H32 also may be expressed by $CD5^+$ CLL B cells with little or no somatic mutation. These findings contrast with a recently published study on V_H5 gene expression in B CLL and contest the hypothesis that extensive somatic mutation is a common property of the V_H5 genes used in this disease. Further work to define the clinical and/or phenotypic characteristics of patients with leukemia cells that express mutated versus nonmutated Ig V genes may reveal subsets of CLL that possibly differ in their cytogenesis, etiopathogenesis, and/or clinical behavior.

B cell chronic lymphocytic leukemia (CLL)[1], the most common adult leukemia in Western societies, may be considered a malignancy of "B-1 B cells", formerly referred to as CD5 (or Ly1) B cells (for reviews see references 1 and 2). Generally, the leukemia cells in this disease coexpress CD5, pan-B cell surface antigens and surface Ig. In addition, common B cell CLL may share additional characteristics with those of normal human B-1 B cells, such as the frequent production of polyreactive autoantibodies (3–5), expression of myelomonocytic surface antigens (6–10), low-level expression of CD20 (11, 12), or the ability to form rosettes with mouse erythrocytes (13, 14).

We found that the leukemia cells from many CLL patients express Ig that bear one or more autoantibody-associated cross-reactive idiotypes (CRIs) (15–18). Evaluation of the molecular basis for such CRIs revealed that each may be a serologic marker for expression of a conserved Ig V gene with limited or no somatic mutation (19–21). In addition, we and others find that CLL B cells not selected for expression of a CRI also may express Ig V genes that share extensive homology (>97%) with other known germline Ig V genes (18, 22–26). Finally, normal or malignant murine B-1 B cells generally also express restricted, if not unique, repertoires of Ig V genes that display little or no somatic mutation (27–30). Collectively, these studies imply that B-1 B cell malignancies use restricted repertoires of Ig V genes that have not diversified substantially from the germline DNA.

However, there is an apparent exception to this generalization. Humphries et al. (31) noted that the leukemia cells from related CLL patients expressed extensively mutated Ig

[1] *Abbreviations used in this paper:* CLL, chronic lymphocytic leukemia; CRI, cross-reactive idiotype; FR, framework region.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/04/1039/08 $2.00
Volume 177 April 1993 1039–1046

V_H genes belonging to the V_H5 subgroup. In addition, they noted that 30% of patients with B cell CLL have rearranged V_H genes that belong to this subgroup. These same investigators examined 11 additional CLL B cell populations that also expressed Ig encoded by V_H5 genes (32, 33). Since prior studies indicated that Ig V_H genes of this small subgroup are highly conserved and nonpolymorphic (34), these investigators compared the primary nucleic acid sequences of the expressed V_H genes with that of known V_H5 genes. In 10 of 11 CLL, they found the expressed V_H5 genes to differ substantially from those of known germline sequences. Importantly, nonconservative nucleic acid base differences were found clustered primarily in sequences encoding the Ig CDRs which form the pocket of the presumed Ig antigen-combining site. Such substitutions often are noted in Ig selected in an antigen-driven secondary immune response. Accordingly, it appears that these CLL express somatically mutated Ig V genes that have been selected for their ability to bind some unknown antigen(s).

It is not certain whether these leukemias represent a distinct subset of CLL or whether they express mutated Ig V gene because they use Ig V_H genes of the V_H5 subgroup. The V_H5 gene subgroup is relatively small, consisting of only two functional genes (V_H251 and V_H32) and one pseudogene (V_H15) (31, 35, 36). The V_H251 gene is distinctive in that it is transcribed at relatively high levels in the germline nonrearranged configuration (36). Conceivably, such transcriptional activity could affect higher rates of Ig gene rearrangement and/or somatic mutation of this V_H gene in CLL B cells or leukemia B cell precursors. Alternatively, despite also being $CD5^+$ (32, 33), the CLL cells examined by these investigators may constitute a discrete subset of chronic leukemia that differs from that of other B cell CLL in its physiology of Ig gene expression. For these reasons, we decided to examine for V_H5 gene rearrangements in the leukemia cells from 68 patients that satisfied diagnostic and clinical criteria for B cell CLL.

Materials and Methods

Patients. Peripheral blood samples were obtained from 68 patients fulfilling diagnostic and immunophenotypic criteria for common B cell CLL at the UCSD Medical Center, the Veteran's Administration Hospital, or the Scripps Clinic and Research Foundation (all in La Jolla, CA; 37–41). The median age of the patients was 63, ranging from 41 to 79 years. The patients were heterogeneous with respect to clinical stage (ranging from Rai stage 0 to stage IV) and prior therapy for CLL. 52 (76%) of the patients were male, reflecting the known 5:2 male/female bias in the prevalence of common B cell CLL (42). Each patient is assigned a unique three-letter identifier (listed in capital letters).

Flow Cytometry. Direct immunofluorescence analyses of leukemia cells were performed using a flow cytometer (FACScan™; Becton Dickinson & Co., San Jose, CA) as described (38). PE- or fluorescein-conjugated mAbs specific for CD20, CD19, or CD5, and their respective isotype control mouse IgG, were purchased from Becton Dickinson & Co. Other mAbs were as described (43).

DNA Isolation and Southern Blotting. Genomic DNA was isolated from the leukemia cells as described (44). A total of 10 μg of DNA was digested with fivefold excess of restriction enzyme in appropriate buffers and then loaded onto a 0.8% agarose gel (GIBCO BRL, Gaithersburg, MD). After electrophoresis for 660 volt-hours, the size-separated DNA was transferred onto nylon for Southern blot hybridization with radiolabeled probes as described (35, 44). Final washing conditions consisted of two 20-min washes at 65°C in 0.2× SSC in 0.1% $NaDodSO_4$ (V_H251 probe), or 0.1× SSC in 0.1% $NaDodSO_4$ (J_H probe) (1× SSC, 0.15 M NaCl/0.015 M sodium citrate, pH 7.0).

Probes and Oligonucleotides. The probe for V_H251 consisted of a 1.0-kb XbaI/PstII fragment of WS1 (35) that included most of the V_H251 coding region along with a few hundred bp of the 5′ flanking region. The J_H probe consisted of a 2.1-kb Sau3A fragment spanning the genomic DNA of J_H2 through J_H6. DNA probes were radiolabeled to a sp act of 10^9 cpm/μg with α-[^{32}P] CTP (New England Nuclear, Boston, MA) via random hexamer priming (45). Oligonucleotides and probes such as the V_H5 third framework region (FR) probe (5′ GCA GAC TTC TCC TCA GG 3′) and the β-globin internal probe (5′ GCA GAC TTC TCC TCA GG 3′) were synthesized as described (15). These probes were 5′ end-labeled using γ-[^{32}P]ATP and polynucleotide kinase to a sp act of 2×10^7 cpm/pmol, as described (46).

PCR Amplification of Genomic DNA. 1 μg of genomic DNA was amplified using the PCR technique as described (44). Primers corresponding to the sense strand of the leader sequence of the V_H251 gene (dGCGAATTCAAGCTTCAACCGCCATCCTCGCC) and the antisense strand of a J_H consensus sequence (dGGCGAATTCACCTGAGGAGACRGTGACC) (R, G/A) were used to amplify V_H5 genes that were juxtaposed to J_H through Ig gene rearrangement. To control for template fidelity, PCR using oligonucleotides dACACAACTGTGTTCACTAGC and dCAACTTCATCCACGTTCACC was performed to amplify the β-globin gene in the genomic DNA of all samples, as described (20).

Cloning and DNA Sequencing. The amplified DNA segments were digested with HindIII and EcoRI to cut the linkers incorporated into the oligonucleotide primers, purified and ligated into a HindIII/EcoRI–cut pUC19 vector (Bluescribe; Stratagene, La Jolla, CA) for transformation of competent *Escherichia coli*, as described (44). Plasmids containing the insert were isolated for double-strand (ds)DNA sequencing as described (47, 48).

Results

Flow Cytometric Analyses. Direct immunofluorescence analyses of the mononuclear cells from each subject substantiated the diagnosis of common B cell CLL, indicating that >70% (mean 92%, range 71–99%) of the lymphocytes in each blood sample coexpressed pan-B cell surface antigens, CD5 and κ or λ L chains (data not shown). Of the 68 leukemia cell populations studied, 39 (57%) expressed Ig κ L chains and 29 (43%) expressed λ L chains.

Southern Blot Analyses. Genomic DNA isolated from each of the 68 samples was digested with BamHI and HindIII for Southern blot hybridization analysis. Hybridization of filter-immobilized DNA with the radiolabeled J_H probe revealed each to have monoclonal Ig gene rearrangements (Fig. 1, and data not shown). 45 (65%) of the samples had two detectable J_H gene rearrangements, whereas the remaining 23 samples each had only one nongermline J_H restriction fragment.

Using the V_H251 probe, we found that most DNA

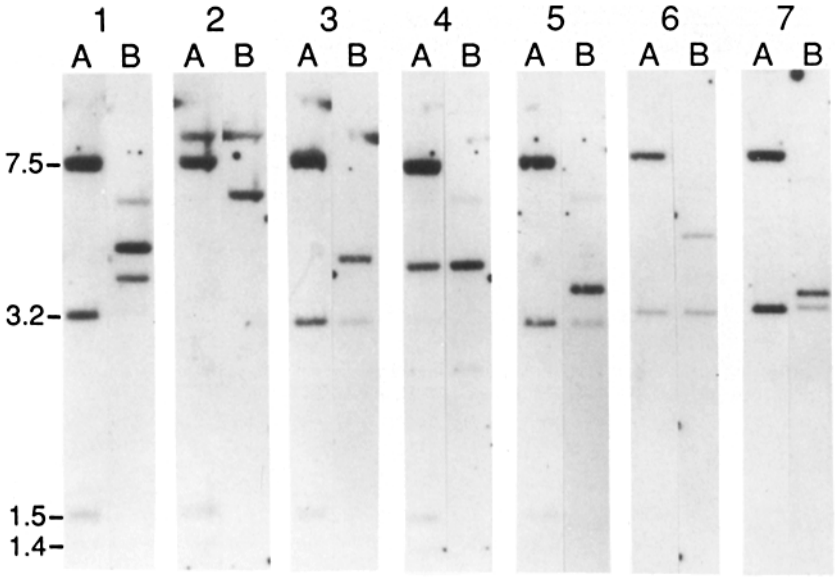

Figure 1. Southern blot analysis of genomic leukemia cell DNA digested with BamHI and HindIII restriction enzymes and hybridized sequentially with radiolabeled probes for V_H251 (lane *A*) and human J_H region (lane *B*). (*Left*) The kb of the nonrearranged bands containing V_H251 (*7.5 kb*), V_H32 (*3.2 kb*), and V_H15 (*1.5/1.4 kb*). (►) The rearranged V_H5 gene identified in each lane *A* of samples 2–7. The samples in each lane are: NOM (lane *1*), HAN (lane *2*), CAV (lane *3*), HOW (lane *4*), PET (lane *5*), ANG (lane *6*), and KER (lane *7*).

samples had the germline V_H5 gene pattern of placental DNA (Fig. 1, lane 1*A*, and data not shown) (31). The V_H251, V_H32, and V_H15 genes are contained in the 7.5-, 3.2-, and 1.4/1.5-kb fragments, respectively (e.g., Fig. 1, lane 1*A* [NOM]). These four bands were identified in 37 (54%) of the 68 samples tested. In 26 (39%) of the samples, the 3.2-kb V_H32 gene fragment was not detected, suggesting that the germline V_H32 genes of both alleles were lost, possibly through Ig gene rearrangement. However, previous studies demonstrated that the germline DNA of many individuals actually lack this 3.2-kb BamHI/HindIII V_H32 restriction fragment (31). As such, the absence of this band in these CLL DNA samples instead may be due to genetic polymorphism.

In 7 (10%) of the 68 CLL DNA samples, we identified nongermline bands with the V_H251 probe. In all cases, these nongermline bands matched those detected using the human J_H probe, arguing that V_H5 genes within these CLL had undergone Ig gene rearrangement (Fig. 1). Two (3% of the total) had rearranged BamHI/HindIII fragments of 8.5 kb, suggesting that these CLL samples each had a rearranged V_H251 juxtaposed to J_H4 (e.g., Fig. 1, lane *2A* [HAN]) (31). In the other five samples, we detected nongermline BamHI/HindIII fragments of 3.0–4.0 kb that matched rearranged bands that hybridized with the human J_H probe (Fig. 1, samples 3 [CAV], 4 [HOW], 5 [PET], 6 [ANG], and 7 [KER]). However, such BamHI/HindIII restriction fragments should be too small to contain a rearranged V_H251 gene juxtaposed with any J_H gene segment, unless alternative BamHI or HindIII restriction sites were introduced into or around this gene through Ig gene rearrangement, somatic mutation, and/or genetic polymorphism. More likely, these relatively small restriction fragments contain V_H32 rearranged with either J_H4 (HOW, Fig. 1, lane *4A*), J_H5 (ANG and KER, Fig. 1, lanes *6A* and *7A*) or J_H6 (CAV and PET, Fig. 1, lanes *3A* and *5A*), respectively.

Sequence Analyses of Rearranged V_H5 Genes. To examine the V_H5 genes juxtaposed to J_H through Ig gene rearrangement, we performed the PCR on the genomic DNA from each of the 68 samples using primers corresponding to the sense strand of the V_H251 leader and the antisense strand of a J_H consensus sequence. We found that only the CLL samples noted to have V_H5 gene rearrangements by Southern analyses yielded PCR products that hybridized with either an oligonucleotide corresponding to the third FR of V_H251 or the genomic V_H251 probe (data not shown). On the other hand, each of the 68 DNA samples yielded 112-bp gene fragments in control PCR using β-globin specific primers (data not shown). Therefore, the inability to amplify V_H5 Ig gene rearrangements in the other DNA samples could not be ascribed to inadequate template quality or nonspecific PCR inhibitors.

Nucleic acid sequence analyses confirmed that each of these seven CLL DNA samples contained rearranged V_H5 genes (Figs. 2 and 3). The two CLL DNA samples reasoned to have possible V_H251 gene rearrangements by Southern (DOB and HAN) were found to have rearranged V_H5 genes that shared ≥98% sequence homology with 5-2R1, a V_H251 gene isolated from an acute pre-B cell leukemia (49) (Fig. 2). The V_H gene of DOB is identical to that of 5-2R1. The V_H of HAN, on the other hand, differs from 5-2R1 by four nucleotides in the first Ig FR and two in the third Ig FR (Fig. 2). However, only two of these base differences are nonconservative, resulting in a Gly → Arg substitution at position 26 and a Ser → Arg change at position 82B (numbering according to Kabat et al. [50]). The rearranged V_H5 genes of the other five CLL (e.g., CAV, HOW, PET, ANG, and KER) were found to share ≥99% nucleic acid sequence homology to a germline V_H32 gene isolated from human sperm DNA (Fig. 2) (31, 51). Except for a conservative base change in the codon for the amino acid at position 7 in the first Ig FR, CAV and HOW are identical to V_H32. The other three V_H genes also differed from V_H32 by one (PET)

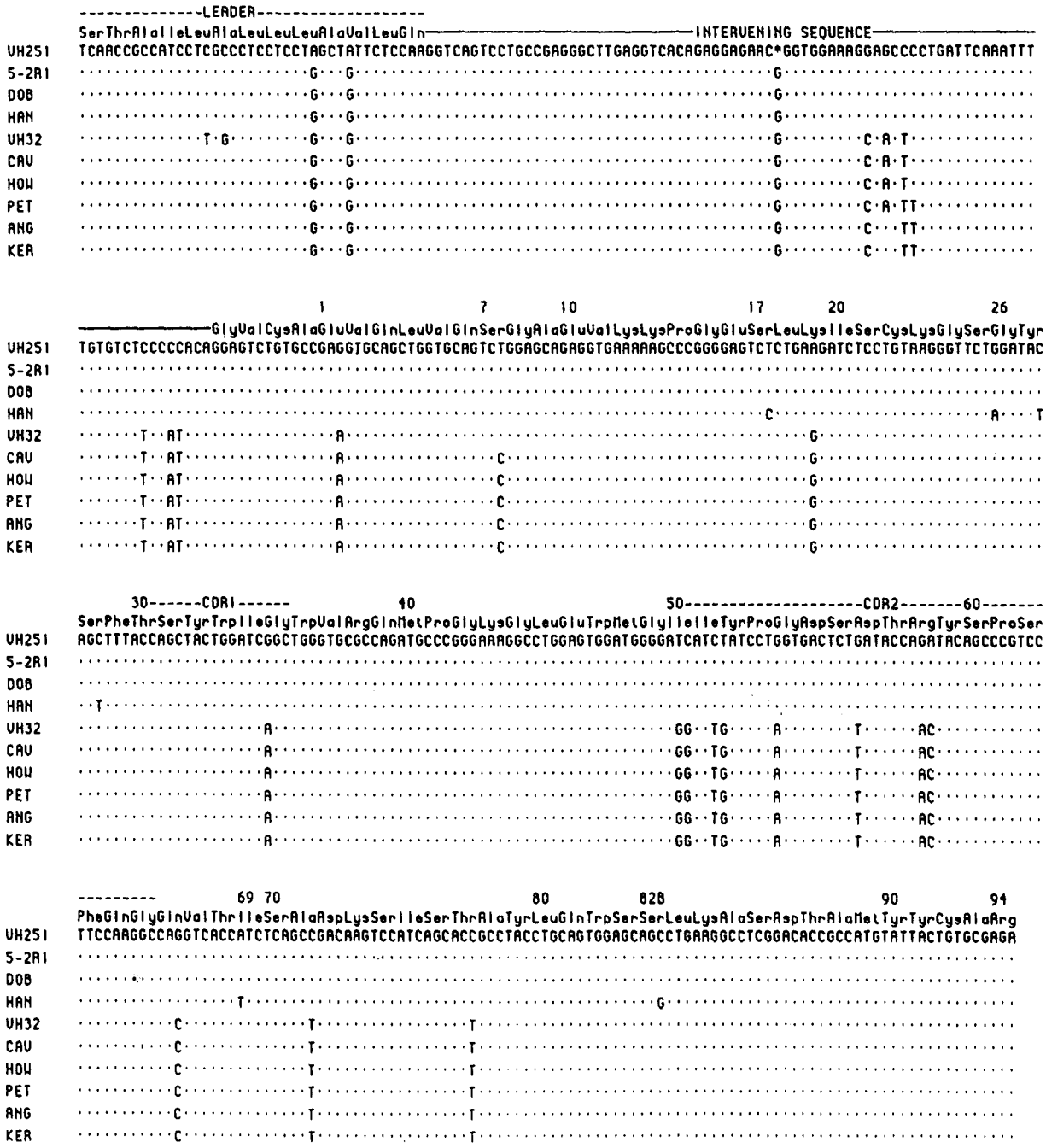

Figure 2. Nucleic acid sequences of rearranged V_H5 genes and V_H32 compared with V_H251. The complete sequence of V_H251 is provided (31). (*) Introduces gaps to maximize sequence homologies among the compared sequences. Above the V_H251 sequence are the deduced amino acid sequence and descriptors indicating the leader sequence, the intervening sequence of the first intron, or the Ig CDR1 and CDR2. The numbers above the deduced amino acid sequence refer to the Ig amino acid residues according to Kabat et al. (50). Below the V_H251 sequence are other V_H5 genes, as indicated by the sequence names (*left*). (·) Indicates homology with V_H251 at a given nucleic acid base position.

or two (ANG or KER) base changes in the intron (marked intervening sequence, Fig. 2).

Each of the rearranged V_H5 Ig genes detected in our survey had a unique sequence encoding the third CDR (CDR3) (Fig. 3). The CDR3 sequence of each had stretches with high homology to known germline D_H and J_H gene segments (50, 52). The sequences encoding the CDR3 of PET, CAV, or HOW each had highest homology with DN1. Those of DOB or KER had the highest homology with Dxp1, whereas the CDR3 sequence of HAN or ANG had the highest homology with DA4 or DK1, respectively. Of the seven disparate CDRs, we deduce that three (DOB, HOW, and HAN)

Figure 3. The sequences encoding the deduced CDR3 of each of the rearranged V_H5 genes compared with germline D_H and J_H minigene sequences having the highest nucleic acid sequence homology. All sequences are identified (*right* and *left*). (*N-D-N*) Column of sequences presumed to be generated from D_H minigenes and N-sequence insertions. (*J_H Segments*) Column of sequences encoded by a rearranged J_H minigene. Above each sequence is the deduced amino acid sequence. The germline D_H and J_H minigenes with highest nucleic acid sequence homology are listed below each sequence. (·) Indicates sequence homology.

were encoded by J_H4, two (ANG and KER) by J_H5, and two (CAV and PET) by J_H6 (Fig. 3).

Discussion

We examined the leukemia cells from 68 randomly selected patients that satisfied clinical and diagnostic criteria for $CD5^+$ B cell CLL. This group of CLL patients was noted to have a mean age and sex distribution comparable with that noted in earlier surveys of patients with this disease (39, 42). Furthermore, FACS® analyses demonstrated that the leukemia cells from each patient coexpressed pan-B cell surface antigens, CD5 and κ or λ Ig L chains. Of these CLL samples, we found that 7 (10%) had Ig gene rearrangements involving the V_H5 gene family. Nucleic acid sequence analyses of these Ig V_H genes did not reveal evidence for intraclonal diversity or Ig somatic mutations. Instead, genetic polymorphism apparently accounts for some of the differences noted in V_H5 genes detected in our survey. For example, the conservative base change in the codon at position 7 of the V_H32 used by some of the CLL (Fig. 2) also was observed in the V_H32 genes of two of three normal individuals examined by Sanz et al. (34). Moreover, all V_H5 genes detected in this survey shared >98% nucleic acid sequence homology with known or suspected germline genes. This paucity of substantial somatic diversification in the rearranged V_H5 genes noted in this study is comparable with that of most other V_H or V_L genes found expressed in this disease (18–26).

In contrast with previous reports on the V_H5 genes rearranged in CLL (31), V_H32 and not V_H251 was the most frequently rearranged V_H5 gene detected in our survey. Initially, V_H32 was assumed to be a pseudogene due to a termination codon at amino acid position 82B (31, 35). However, Sanz et al. (34) reexamined the original V_H32 clones and found errors in the reported sequence, allowing these investigators to conclude that V_H32 was in fact functional. The rearranged V_H32 genes observed in the current survey also lack any termination codons and appear functional. In view of the finding that many individuals apparently lack the V_H32 germline band, the relatively frequent use of V_H32 in CLL appears remarkable. Indeed, the absence of the V_H32 germline band may indicate that a relatively high proportion of individuals do not possess this Ig V gene in their germline DNA. However, other explanations are possible, including polymorphisms affecting the restriction sites in and around V_H32 and, at least in the current survey, Ig gene rearrangements and/or deletions by the leukemia cells studied.

In any case, it is unlikely that we failed to detect rearranged V_H251 genes in our survey that incurred extensive and selected somatic mutations. We performed Southern blot analyses using washing conditions that allow for detection of rearranged V_H genes that share >80% homology with V_H251.

In addition, over half of the V_H251 probe used in these analyses corresponded to the 5′ flanking region and intron of the V_H251 gene, regions not subject to selected Ig somatic mutation. Furthermore, using the V_H251 probe we detected both V_H32 and V_H15, V_H5 genes having 95 and 86% coding region nucleic acid sequence homology with V_H251, respectively. Finally, PCR on all leukemia samples using oligonucleotides to amplify V_H5 genes juxtaposed with J_H demonstrated that only those DNA samples that had V_H5 gene rearrangement by Southern also generated PCR products that hybridized with oligonucleotides or full-length DNA probes for V_H251 and related V_H5 genes.

These results contrast markedly with those recently reported by Cai et al. (32, 33), indicating that highly mutated V_H251 genes frequently are expressed in human B cell CLL. Earlier reports indicated that V_H251 gene may be rearranged in the leukemia cells of 30% of the patients with this disease (31). More importantly, nucleic acid sequence analyses revealed extensive numbers of base substitutions from the putative germline V_H251 sequence. Moreover, the noted pattern of nonconservative base substitutions in the V_H251 gene used by these CLL B cells was typical of Ig V genes expressed in secondary immune responses to antigen (32, 33). In contrast, we found that only 2 of 68 CLL cell samples use V_H251, and that these two apparently express this gene with little or no somatic mutation.

Such differences may reflect heterogeneity within what currently is considered B cell CLL. Subgroups of CLL may exist that arise from different B cell lineages and/or stages of B cell differentiation. Such differences may be reflected in the Ig V genes used by each subgroup. Already, we have noted that $CD5^-$ B cell CLL, in contrast with $CD5^+$ B cell CLL, may display intraclonal diversity in their expressed Ig V genes (25, 53). Although both the current survey and that of Cai et al. (32, 33) examined $CD5^+$ B cell CLL, there also may exist subgroups within this category of CLL that express Ig similar to that found in the primary (e.g., nonmutated) versus secondary (e.g., mutated) humoral immune response. Arguably, the leukemia cells in the former category may be those that express "natural" or polyreactive autoantibodies that frequently are detected in $CD5^+$ B cell CLL (3–5). On the other hand, those CLL in the latter category may originate from B cells previously selected to express Ig with affinity for some unknown environmental or self-antigen(s). Antigen selection may not be unique to this subgroup, however. Recent Ig chain mixing studies employing murine transfectomas have indicated that the polyreactive binding activity of Ig encoded by nonmutated Ig V genes also may be a selected specificity (54). In addition, as demonstrated in this survey, it is not likely that the observed differences in the extent of observed Ig mutation depends upon expression of Ig V genes of the V_H5 subgroup, in particular V_H251. Rather, there may exist subsets of $CD5^+$ B cell CLL that have differences in their cytogenesis and/or etiopathogenesis. Comparison of B cell CLL that express nonmutated versus highly mutated Ig V genes may reveal features that can be used to discriminate between these two types of leukemias, possibly allowing us to define subsets of CLL that have different clinical features and/or therapeutic requirements.

The authors appreciate the excellent technical assistance of Mr. Todd Johnson and Mr. Grant Meisenholder.

This work was funded in part by research grants AG-04100 and CA-49870 from the National Institutes of Health.

Address correspondence to Dr. Thomas J. Kipps, Division of Hematology and Oncology, Department of Medicine, and the Sam & Rose Stein Institute for Research on Aging, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093–0663.

Received for publication 13 November 1992.

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